DESCRIPTION

Alkaline Protease

Technical Field

The present invention relates to an alkaline protease useful as an enzyme incorporated in a detergent; a gene encoding the same; a microorganism producing the same; and a detergent composition containing the same.

Background Art

Protease has been widely used in a variety of detergents, such as laundry detergents; cosmetic compositions; bath additives; food-modifying agents; and pharmaceuticals such as digestive aids and antiphlogistics.

Of these, proteases used in detergents are produced in largest amounts on an industrial scale and thus account for a significant part of commercial supply. Examples of such proteases include Alcalase, Savinase (product of Novo Nordisk), Maxacal (product of Genencor), Blap (Product of Henkel), and Protease K (KAP, product of Kao Corporation).

Meanwhile, attempts have been made to improve the performance of enzymes used in detergents. For example, Japanese Patent Application Laid-Open (*kokai*) No. 6-70765 discloses an enzyme having high stability to heat and a surfactant. Japanese Patent Application Laid-Open (*kokai*) No. 9-121855 discloses an enzyme which acts on insoluble proteins

such as keratin and has a high specific activity. Japanese Patent Application Laid-Open (*kokai*) Nos. 5-211868 and 9-121856 disclose an enzyme having excellent activity in a low temperature range. European Patent No. 0130756 discloses a method for enhancing stability of an enzyme to an oxidizing agent.

In many cases, soils on laundry comprise a plurality of components such as lipids and solid particles other than protein. Therefore, there is demand for a detergent having excellent detergency to such complex soils. In order to meet the demand, generally a plurality of enzymes and surfactants have been incorporated into a detergent.

However, even though a plurality of enzymes are incorporated, their effects cannot be fully exerted if, in the presence of complex soils, the enzymes are unstable and do not exhibit constant and sufficient activity. Conventional enzymes are unsatisfactory in this point.

Disclosure of the Invention

In view of the foregoing, the present inventors have discovered an alkaline protease which has a constant casein-degrading activity even in the presence of a fatty acid at a high concentration and exhibits excellent detergency even under complex soil conditions; e.g., soils containing protein and sebum.

Accordingly, in one aspect of the present invention, there is provided an alkaline protease which has the

following physicochemical properties:

(i) Acting pH range

acting over a wide pH range of 4-13 and exhibiting, at a pH of 6-12, 80% or more the activity at the optimum pH;

(ii) Stable pH range

being stable over a pH range of 6-11 when treated at 40°C for 30 minutes;

(iii) Isoelectric point

having an isoelectric point of approximately 8.9-9.1; and

(iv) Effect of a fatty acid

casein-degrading activity not being inhibited by oleic acid.

In another aspect of the present invention, there is provided a gene encoding the above-described alkaline protease.

In still another aspect of the present invention, there is provided a microorganism producing the above-described alkaline protease.

In yet another aspect of the present invention, there is provided a detergent composition containing the above-described alkaline protease.

Brief Description of the Drawings

Fig. 1 shows the the effects of pH on the activity of alkaline protease KP43. Fig. 2 shows the effects of pH on the stability of alkaline protease KP43 (40°C, 30 minutes).

Fig. 3 shows the effects of pH on the stability of alkaline protease KP43 (10°C, 24 hours). Fig. 4 shows the effects of temperature on the activity of alkaline protease KP43. Fig. 5 shows the effects of temperature on the stability of alkaline protease KP43. Fig. 6 shows the effect of an oxidizing agent (50 mM hydrogen peroxide) on the activity of alkaline protease KP 43. Fig. 7 shows N-terminal sequences of KP9860 protease and partially degraded products thereof. Fig. 8 shows primer sequences designed from an N-terminal sequence of KP9860 protease. Fig. 9 shows 57 bp PCR-amplified fragments and primer designs.

Best Mode for Carrying Out the Invention

The alkaline protease of the present invention has the above-described physicochemical properties (i) through (iv). Of these, property (iv) is particularly important. The alkaline protease has a casein-degrading activity in the presence of 10 mM of oleic acid, a component of sebum, as high as that in the absence of oleic acid.

The alkaline protease of the present invention preferably has (v) an estimated molecular weight of approximately 43,000 as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Particularly preferred is an alkaline protease having, in addition to properties (i) through (v), properties (vi) through (ix) as described below.

(vi) Acting temperature and optimum temperature

acting at an optimum temperature of 60°C-70°C, and also acting at a temperature as low as 20°C or lower;

(vii) Effects of metal ions

activity being inhibited by Hg^{2+} and Cu^{2+} and thermal stability being enhanced by Ca^{2+};

(viii) Effects of inhibitors

activity not being inhibited by ethylenediaminetetraacetic acid (EDTA) and p-chloromercurybenzoic acid (PCMB) and activity being inhibited by diisoproyl fluorophosphate (DFP) and phenylmethanesulfonyl fluoride (PMSF); and

(ix) Effects of surface active agents

activity not being inhibited by linear sodium alkylbenzenesulfonate, sodium polyoxyethylene alkyl sulfate, sodium dodecyl sulfate, sodium α-olefinsulfonate, or α-sulfofatty acid ester.

The alkaline protease of the present invention preferably has an amino acid sequence shown by Sequence No. 1 or 2, or such a sequence in which one or more amino acids are deleted, substituted, or added. Sequence No. 1 differs from Sequence No. 2 in that lysine at the 3rd position in Sequence No. 2 is deleted. Xaa in Sequence Nos. 1 and 2 refers to an arbitrary amino acid. Preferable amino acids for Xaa at each position in Sequence No. 2 are shown in the following Table.

Table

position		Position	
24	Ser or Asn	30	Gly or Asp
33	Asn or Thr	47	Ala or Val
48	Lys or Ser	54	Gly or Arg
71	Pro or Leu	75	Gln or Leu
90	Ile or Val	103	Gln or Lys
106	Lys or Thr	129	Lys or Gln
131	Ala or Lys	132	Thr or Val
133	Ser or Arg	134	Thr or Ser
147	Ile or Lys	149	Arg or Lys
161	Glu or Thr	166	Val or Leu
173	Lys or Asn	184	Gln or Glu
188	Phe or Tyr	189	Ala or Val
190	Ile or Ala	195	Leu or His
287	Ser or Ala	307	Gly or Ser
325	Tyr or Phe	370	Gly or Arg
432	Phe or Tyr	502	Ile or Val
532	Ser or Ala	542	Ser or Thr
585	Gln or Arg	592	Thr or Ser
593	Ser or Ala	595	Tyr or Phe
596	Asn or Asp	597	Asp or Asn
612	Ala or Ser	633	Thr or Asn

Deletions, substitutions, and additions in the alkaline protease of the present invention are not particularly limited. However, the amino acid sequence shown in Sequence No. 1 or 2 is preferably conserved in the amount of 70% or more, more preferably 80% or more, particularly preferably

90% or more.

Examples of the alkaline proteases include alkaline proteases having an amino acid sequence shown by Sequence No. 3, 4, or 5, or such a sequence in which one or more amino acids are deleted, substituted, or added.

The alkaline protease of the present invention may be produced by cultivating alkaline protease-producing microorganisms belonging to the genus *Bacillus* and collecting the enzyme from the culture broth. Examples of alkaline protease-producing microorganisms according to the present invention include wild strains belonging to the genus *Bacillus* and a transformant containing a gene encoding a peptide having the above-described amino acid sequence. Examples of the wild strains include KP-43, KP-1790, and KP-9860. Mycological characteristics of these strains are shown below.

Table 1-a

	KP43	KP1790	KP9860
A. Morphological characteristics			
(a)Gram's staining	positive	positive	positive
(b)Aminopeptidase	undefined	undefined	undefined
(c)Movement	yes	yes	yes
(d)Flagella	peritrichous flagella	peritrichous flagella	peritrichous flagella
(e)Spores (type, shape, site, swell)	sporogenous, eliptical, central, none	sporogenous, eliptical, central, none	sporogenous, eliptical, central to terminal, swollen
B. Physiological characteristics			
(a)Nitrate reduction	negative	negative	negative
(b)Production of indole	negative	negative	negative
(c)Growth pH range	can grow at pH 6.2-11.7, well grow at pH 8-10	can grow at pH 6.2-11.7, well grow at pH 8.5-10	can grow at pH 6.2-10.0, well grow at pH about 9
(d) Resistance to sodium chloride	cannot grow under ≥7% NaCl	cannot grow under ≥7% NaCl	cannot grow under ≥7% NaCl
(e)Growth temperature range	10-40° C	10-40° C	20-40° C
(f)β-Galactosidase	positive	positive	positive
(g)Arginine dihydrolase	negative	negative	negative
(h)Lysine dihydrolase	negative	negative	negative
(i)Oxydase	positive	positive	positive
(j)Utilization of citric acid	negative	negative	negative
(k)Utilization of urea	negative	negative	negative
(l)Catalase	positive	positive	positive
(m)Gas production from glucose and nitrate	negative	negative	negative
(n)Growth under anaerobic conditions	negative	negative	negative
(o)V-P test	negative	negative	negative

(continued to Table 1-b)

Table 1-b

	KP43	KP1790	KP9860
(p)Acid production from sugar			
D-Glucose	+	±	+
L-Arabinose	-	-	-
D-Xylose	-	-	-
D-Mannitol	+	+	+
D-Galactose	±	-	-
Sucrose	+	+	+
D-Mannose	+	±	+
Inositol	-	-	-
D-Sorbitol	+	-	-
Trehalose	±	+	+
Lactose	-	-	-
Glycerol	-	-	-
Maltose	+	±	+
D-Fructose	+	+	+
Raffinose	-	-	-
Melibiose	+	-	-
Starch	+	+	+

Based on the above-described mycological characteristics, the three strains were examined by reference to the pertinent descriptions in "Bergey's Manual of Systematic Bacteriology" (Williams & Wilkins Co., 1984), and were considered to belong to the genus *Bacillus*. However, these strains are novel microorganisms in that characteristics of these species do not completely match those of known species belonging to the genus *Bacillus*. Thus, the three strains were deposited with National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, 305-0046, JAPAN) as *Bacillus sp.* KSM-KP43 (FERM BP-6532), *Bacillus sp.* KSM-KP1790 (FERM BP-6533), and *Bacillus sp.* KSM-

KP9860 (FERM BP-6534) (Date of original deposit: September 18, 1996).

In order to produce the alkaline protease of the present invention by use of the above-described strains, the strains are inoculated in a medium containing an assimilablecarbon source, a nitrogen source, and essential nutrients and are cultured through a customary method.

Collection and purification of a target alkaline protease from the thus-obtained culture broth can be performed according to conventional methods applicable to the collection and purification of common enzymes. For example, cells are separated from the culture broth by centrifugation or filtration, and the target alkaline protease can be obtained from the supernatant through a customary purification method. The thus-obtained enzyme liquid may be used as such or may be further purified and crystallized through a known method.

Alternatively, the alkaline protease of the present invention may be produced through the following steps: obtaining a gene encoding the alkaline protease; preparing a recombinant vector by use of the gene; transforming a host cell by use of the recombinant vector; cultivating the obtained transformant; and collecting the target alkaline protease from the cultured product.

The gene encoding the alkaline protease of the present invention may be cloned from any of the three above-described strains. Cloning may be performed through known methods.

Examples of the methods include (1) the shot gun method comprising preparation of a DNA fragment through complete or partial digestion of chromosomal DNA by use of an appropriate restriction endonuclease; combination of the fragment into a suitable vector; and expression through introduction to *Escherichia coli* or *Bacillus subtilis*, and (2) a method comprising synthesis of an appropriate primer and cloning a target gene through PCR.

Examples of the nucleotide sequence of the alkaline protease of the present invention are shown in Sequence Nos. 3 to 5. The nucleotide sequence is not limited to Sequence Nos. 3 to 5, and acceptable sequences may include a nucleotide sequence encoding the amino acid sequence shown in Sequence No. 1 or 2, and a nucleotide sequence encoding such an amino acid sequence in which one or more amino acids are deleted, substituted, or added. Of these, nucleotide sequences represented by Sequence Nos. 3 to 5, or such sequences in which one or more amino acids are deleted, substituted, or added are preferred. In these cases, deletion, substitution, or addition preferably occurs within the above-described variation of amino acid sequence.

In order to prepare a recombinant vector including the above-described gene encoding an alkaline protease, the gene may be incorporated into an arbitrary vector suitable for expression of the gene in a host of interest. Examples of the vectors include pUC18, pBR322, and pUC19 in the case in which *Escherichia coli* serves as a host and pUB110 in the

case in which *Bacillus subtilis* serves as a host.

A host is transformed by use of the thus-obtained recombinant vector through a customary method such as the protoplast method or the competent cell method. Although no particular limitation is imposed on the host, microorganisms are preferred. Examples include Gram-positive bacteria such as microorganisms belonging to the *genus Bacillus*, Gram-negative bacteria such as *Escherichia coil*, yeast belonging to *Saccharomyces*, and fungus belonging to *Aspergillus*.

In order to produce the alkaline protease of the present invention through culturing of the obtained transformant, cultivation, collection, and purification may be performed in accordance with a procedure employed in the case in which the above-described wild strain is used.

As described above, the alkaline protease of the present invention has excellent resistance to alkaline conditions and excellent protease activity even in the presence of lipids. Thus, the alkaline protease is useful for an enzyme incorporated in a variety of detergent compositions.

No particular limitation is imposed on the amount of the above-described alkaline protease incorporated into a detergent composition, and the amount is preferably 0.1-5000 U based on 1 kg, particularly preferably 1-500 U, of the detergent composition.

Known detergent components may be incorporated into the detergent composition of the present invention containing the

alkaline protease. For example, components described in WO94/26881 (p. 5, upper-right column, line 14 - lower-right column, line 29) may be employed.

A surfactant is incorporated into the detergent composition in an amount of 0.5-60 wt.% (hereinafter simply referred to as "%"), particularly preferably 10-45%, into a powdery detergent composition and in an amount of 20-50% into a liquid detergent composition. When the detergent composition of the present invention serves as a bleaching detergent composition or a detergent composition for an automated dishwasher, a surfactant is typically incorporated in an amount of 1-10%, preferably 1-5%.

A divalent metal ion scavenger is incorporated in an amount of 0.01-50%, preferably 5-40%.

An alkali agent and an inorganic salt are incorporated in an amount of 0.01-80%, preferably 1-40%.

An anti-redeposition agent is incorporated in an amount of 0.001-10%, preferably 1-5%.

The detergent composition may contain an enzyme other than the alkaline protease of the present invention. Examples include cellulase, amylase, protopectinase, pectinase, lipase, hemicellulase, β-glucosidase, glucose-oxidase, and cholesterol-oxidase. These enzymes are incorporated in an amount of 0.001-5%, preferably 0.1-3%.

A bleaching agent such as hydrogen peroxide or percarbonate is preferably incorporated in an amount of 1-10%. When a bleaching agent is incorporated, a bleach-activator

may be incorporated in an amount of 0.01-10%.

Examples of fluorescent agents incorporated into the composition include a biphenyl compound, such as Cinopearl CBS-X, and a stilbene compound such as DM-type fluorescent agent. The fluorescent agent is preferably incorporated in an amount of 0.001-2%.

The above-described detergent composition may be processed into a variety of forms such as liquid, powder, and granules. The detergent composition may be used for laundry, an automated dishwasher, drain pipes, and dentures, and may be used as a bleaching agent.

Examples

Example 1 (screening for alkaline protease-producing microorganisms)

A soil sample (1 g) was suspended in physiological saline (10 ml) and thermally treated at 80°C for 10 minutes, followed by inoculation in liquid enrichment medium for protease-producing microorganisms, the medium having the following composition, to thereby culture at 20°C. After subculture enrichment was repeated about three times in the same medium, the cultivated product was smeared onto a plate for judging protease-production and cultivated at 20°C for 5-7 days. Colonies around which a transparent zone was formed by dissociation of skim milk were selected for collection of protease-producing microorganisms. By means of the above procedure, the Bacillus sp KSM-KP43 strain, the KSM-KP1790

strain, and the KSM-KP9860 strain were obtained as alkaline protease-producing microorganisms.

Table 2

Composition of liquid enrichment medium for screening (pH 11)

Monopotassium phosphate	0.1%
Magnesium sulfate	0.02%
Yeast extract (Difco)	0.05%
Keratin (Tokyo Kasei)	1.0%
Glucose	0.5%
Sodium carbonate	0.3%

Agar plate medium for screening

Nutrient agar (Difco)	2.3%
Skim milk (Difco)	0.3%
Sodium carbonate	1.0%

Example 2

The Bacillus sp KSM-KP43 strain obtained in Example 1 was inoculated in a liquid medium comprising polypeptone S (1%), yeast extract (0.05%), potassium phosphate (0.1%), magnesium sulfate (0.02%), glucose (separately sterilized) (1%), and sodium carbonate (separately sterilized) (0.5%) to thereby be cultivated at 30°C for 24 hours. The concentration of enzyme in the supernatant liquid was about 1.5 U/L. The supernatant liquid which had been centrifugally separated from cells at 4°C was added with pulverized ammonium sulfate under stirring so as to attain 90% of

saturated concentration. The solution was maintained under stirring at 4°C for an entire day and night and the resultant precipitate was centrifugally collected. The obtained precipitate was dissolved in 10 mM of a Tris-hydrochloric acid buffer solution (pH 7.5) containing 5 mM of calcium chloride, followed by dialysis through the buffer solution. Subsequently, the dialyzed liquid was applied to a DEAE-Sepharose FF column (product of Pharmacia) which had been equilibrated with 10 mM of a Tris-hydrochloric acid buffer solution (pH 7.5) containing 5 mM of calcium chloride, to thereby collect the non-absorbed fraction. The fractionated liquid was dialyzed through 50 mM of HEPES buffer solution (pH 7.5) containing 2 mM of calcium chloride and was applied to a SP-Sepharose FF column which had been equilibrated with the same buffer solution, to thereby collect an active fraction which has eluted slightly after the non-absorbed fraction. While the active fraction, which had a recovery ratio of 15%, was used as a sample, SDS-polyacrylamide electrophresis was carried out, and as a result, a single band was obtained for the respective enzyme.

Example 3

The obtained Bacillus sp KSM-KP1790 strain and KSM-KP9860 strain were cultivated in the same medium as in Example 2 and the alkaline protease was purified in the same manner as in Example 2.

Example 4

Enzymatic properties of the alkaline proteases obtained in Example 2 and 3 were examined. The methods and results of the experiments are described below.

I. Materials and methods for experiments

(1) Methods for activity measurement

(a) Method in which casein is used as a substrate

After 1 mL of 50 mmol/L of various buffer solutions containing 1% (w/v) Casein (Hammerstein: product of Merck Inc.) was maintained at 40°C for 5 minutes, 0.1 mL of an enzyme solution was added to the solution, followed by incubation at 40°C for 10 minutes. 2 mL of a TCA solution (0.11 mol/L trichloroacetic acid : 0.22 mol/L sodium acetate : 0.33 mol/L acetic acid) was added to stop the reaction and the mixture was left to stand at room temperature for 10 minutes. Subsequently, acid-denatured protein was filtered (No. 2 filter paper: product of Whattmann). To 0.5 mL of the filtrate, 2.5 mL of alkaline copper reagent (1% (w/v) sodium potassium tartrate : 1% (w/v) copper sulfate : 2% (w/v) sodium carbonate, 0.1 mol/L sodium hydroxide = 1:1:100 (v/v)) was added, and after the solution was maintained at 30°C for 10 minutes, 0.25 mL of diluted phenol reagent (phenol reagent (product of Kanto Chemical) diluted two-fold with deionized water) was added, and after being maintained at 30°C for 30 minutes, the solution was subjected to an absorbance measurement at 660 nm. The following solution was used as a blank: to the above-

described system of enzyme reaction, a reaction termination solution was mixed and then the enzyme solution was added.

One unit (P.U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(b) Method in which Synthetic oligo-peptide is used as a substrate

0.05 mL of 50 mmol/L synthetic oligo-peptide solution (succinyl-alanyl-alanyl-prolyl-leucine para-nitroanilide dissolved in dimethyl sulfoxide) was mixed into 0.9 mL of 100 mmol/L boric acid buffer solution (pH 10.0, containing 2 mmol/L of calcium chloride), and after the solution was maintained at 30°C for 5 minutes, 0.05 mL of an enzyme solution was added, followed by incubation at 30°C for 10 minutes. 2 ml of 5% (w/v) citric acid was added to stop the reaction and absorbance at 420 nm was measured.

One unit (U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(c) Method in which Hemoglobin is used as a substrate

According to the method by Anson (M. L. Anson, J. Gen. Physiol. 22, 79(1983)), hemoglobin of bovine blood serum was denatured by use of urea and adjusted to pH 10.5 with sodium hydroxide. 0.1 mL of an enzyme solution (1.0×10^{-5} - 1.0×10^{-3} A.U) was added to 0.5 mL of the substrate solution (2.2%

in terms of hemoglobin), and the resultant solution was incubated at 25°C for 10 minutes. To the resultant solution, 1.0 mL of 4.9% tirchloroacetic acid was added to stop the reaction. After completion of the reaction, centrifugation (3,000 rpm, 10 minutes) was carried out and protein degradation products in the supernatant liquid were quantitatively determined according to the Folin-Lowry method (O. H. Lowry *et al.*, J. Biol. Chem., 193, 265(1951)).

One unit (A. U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(2) Optimum pH

0.1 mL of an enzyme solution (3.0×10^{-5} mP. U) was added to 1 mL of 50 mmol/L Britton-Robinson buffer solution containing 1% (w/v) casein, and activity was measured according to the casein method.

(3) pH stability

An enzyme solution (8.0×10^{-4} mP. U.) was mixed into Britton-Robinson buffer solution (20 mmol/L, containing 2 mmol/L calcium chloride), followed by treatment at 40°C for 30 minutes or at 10°C for 24 hours. After ice-cooling, the treated solution was diluted 40-fold with 50 mmol/L boric acid buffer solution, followed by measurement of residual activity according to the method in which casein is used as a substrate.

(4) Optimum temperature

0.1 mL of the enzyme solution (2.0×10^{-5} mP. U.) was added to 1 mL of 50 mmol/L boric acid buffer solution (pH 10.0) containing 1% (w/v) casein, and activity of the enzyme was measured at temperatures between 10-80°C according to the casein method.

The activity measurements were conducted in both systems; i.e., in the presence of and in the absence of 5 mmol/L calcium chloride.

(5) Heat stability

An enzyme solution (2.5×10^{-4} mP. U.) was added to 20 mmol/L boric acid buffer solution (pH 10.0) in both systems; i.e., in the presence of and in the absence of 5 mmol/L calcium chloride, and thermally treated at the appropriate temperature for 10 minutes. After being cooled with ice, the treated solution was diluted 5-fold with 50 mmol/L boric acid buffer solution (pH 10.0), and residual activity was measured using casein as a substrate.

(6) Effects of metal ions

An enzyme solution (4.0×10^{-4} mP. U.) was added to 20 mmol/L boric acid buffer solution (pH 10.0) containing 1 mmol/L various metal salts, and the resultant solution was incubated at 30°C for 20 minutes. The solution was diluted 5-fold with 50 mmol/L boric acid buffer solution (pH 10.0), followed by measurement of activity using casein as a substrate.

(7) Effects of inhibitors

The enzyme solution (1.0×10^{-3} mP. U.) was added to 10

mmol/L phosphoric acid buffer solution (pH 7.0) containing various inhibitors so as to attain a predetermined concentration, and the solution was incubated at 30°C for 20 minutes. Subsequently, the solution was diluted 20-fold with deionized water, and residual activity was measured using casein as a substrate.

(8) Effects of surfactants

An enzyme solution (7.0×10^{-4} mP. U.) was added to 100 mmol/L boric acid buffer solution containing dissolved surfactants in an amount of 1%, and the resultant solution was incubated at 40°C for 4 hours. The solution was diluted 20-fold with 50 mmol/L boric acid buffer solution (pH 10.0), and residual activity was measured using casein as a substrate.

(9) Effects of oxidizing agent (hydrogen peroxide)

2.7 mL of Britton-Robinson buffer solution containing hydrogen peroxide and calcium chloride (final concentration: 50 mmol/L hydrogen peroxide, 2 mmol/L calcium chloride, 20 mmol/L Britton-Robinson) (pH 8.0) was maintained at 30°C for 15 minutes, and then 0.3 mL of an enzyme solution was added. With the passage of time, 0.8 mL of the resultant solution was sampled in a previously prepared test tube containing 5 μL of catalase (Boehringer Mannheim Co.: 20 mg/L), to thereby stop the oxidation reaction. Each sample was suitably diluted with 2 mmol/L calcium chloride, and residual activity was measured according to the method in which synthetic oligo-peptide is used as a substrate.

(10) Effects of fatty acids

By use of 50 mM phosphoric acid buffer solution (pH 7) containing 1% (w/v) casein as a substrate solution, a reaction was carried out in the presence of 0-10 mM sodium oleate at 20°C for 15 minutes, and activity was measured using casein as a substrate.

II. Results

(1) Optimum pH

Effects of pH on three kinds of protease (KP43, KP1790, and KP9860) were examined. Fig. 1 shows the activities of KP43 at each pH value normalized with respect to activity at optimum pH (100%), indicating that the optimum working pH range of the proteases of the present invention is 6-12. Thus, these enzymes exhibit a high protein-degradation activity in the extensively broad working pH range.

(2) pH stability

After being allowed to stand at 40°C for 30 minutes or at 10°C for 24 hours, the residual activity of KP43 was measured over a range of pH values. Figs. 2 and 3 show the residual activities normalized with respect to the enzyme activity before treatment (100%). The results show that the enzymes of the present invention are stable over the pH range of 6-12 after treatment at 40°C for 30 minutes, and that addition of calcium ions improves enzyme stability at pH 5. Similarly, the results show the enzymes of the present invention are stable over the broad pH range of 5-12 after treatment at 10°C for 24 hours.

(3) Optimum temperature

By use of casein as a substrate, the effects of temperature on the proteases were examined. Fig. 4 shows the activities of KP43 over a range of temperatures, normalized with respect to the highest activity in the absence of calcium ions (100%). The results indicate that in the absence of calcium ions the optimum temperature is 60°C, and in the presence of calcium ions the optimum temperature is 70°C for all three kinds of proteases. Therefore, the results show that the optimum temperature is shifted upward by addition of calcium ions, as is the case with conventional proteases for a detergent.

(4) Heat stability

Heat treatment was carried out for 10 minutes at temperatures in the range of 30-80°C (pH 10.0, in the presence of and in the absence of 5 mmol/L calcium chloride), and residual activity was measured. Fig. 5 shows residual activity of KP43 at each treatment temperature, normalized with respect to the activity before treatment (100%). The results indicate that the proteases are stable at the temperature up to 60°C in the absence of calcium chloride, and that addition of calcium chloride (5 mmol/L) has the effect of shifting temperature stability upward about 10°C. In comparison with commercially available detergent enzymes, these enzymes have high temperature stability; namely, stability comparable to that of Esperase, which exhibits the most excellent temperature stability among commercially

available enzymes.

(5) Effects of metal ions

In 20 mmol/L boric acid buffer solution (pH 10), 3 kinds of proteases were treated with various metal salts (1 mmol/L) at 30°C for 20 minutes and the residual activity was measured. Residual activity is normalized with respect to enzyme activity obtained for protease treated in the same manner except without the addition of metal salts (100%) (see Table 3.) The results show that the activity is inhibited by mercury chloride and silver nitrate but that the activity is extremely stable for other metal salts.

Table 3

Metal salt (1 mM)	Residual activity (%) KP43	KP1790	KP9860
not added	100	100	100
$AgNO_3$	66	70	45
$NiCl_2$	92	95	96
$CaCl_2$	97	95	101
$CoCl_2$	91	101	98
$FeCl_3$	93	113	96
$ZnCl_2$	85	94	91
$CuCl_2$	91	96	94
$HgCl_2$	38	37	33
$MgCl_2$	92	103	100

Treatment conditions: 1 mM metal salt, 20 mM borate buffer (pH 10.0) 30°C, 20 minutes

(6) Effects of various inhibitors

Effects of general enzyme inhibitors on the alkaline proteases of the present invention were examined. A variety of inhibitors were added to 10 mmol/L phosphoric acid buffer solution (pH 7.0) so as to attain the predetermined concentration, and the resultant solution was incubated at

30°C for 20 minutes, after which residual activity was measured. The residual activity is normalized with respect to the enzyme activity obtained for protease treated in the same manner as described above in the absence of inhibitors (100%) (refer to Table 4). The results indicate that for all three kinds of proteases activity was inhibited by diisopropyl fluorophosphoric acid (DFP), phenylmethanesulfonyl fluoride (PMSF), and chymostatin, which are known inhibitors of serine protease. Therefore, the proteases of the present invention are considered to have serine residue in its active center. In contrast, effects of actinomycetes-derived antipine and leupeptin, which has been reported to inhibit serine protease, were not found.

Table 4

Inhibitor	Residual activity (%)			
	Concentra-tion(mM)	KP43	KP1790	KP9860
free	-	100	100	100
EDTA	5	110	97	101
EGTA	5	92	91	90
o-Phenanthroline	5	100	103	100
DTT	5	104	102	105
PCMB	1	125	115	126
NEM	5	97	100	100
DFP	1	14	17	16
PMSF	1	0	0	0
Chymostatin	0.1	87	87	80
Antipine	0.1	103	99	97
Leupeptin	0.1	102	101	93
E-64	0.1	104	99	103
Elastatinal	0.1	99	102	102

EDTA: ethylenediaminetetraacetic acid (Sigma)
EGTA: ethyleneglycoltetraacetic acid (Sigma)
DTT : dithiothreitol (Sigma)
PCMB: p-chloromercury benzoate (Sigma)

NEM : N-ethylmaleimide (Sigma)
DFP : diisopropylfluorophosphoric acid (Sigma)
PMSF: phenylmethanesulfonyl fluoride (Sigma)

(7) Effects of surface active agents

Each protease was treated with a variety of 1% surface active agent at 40°C for 4 hours in 0.1 mol/L Tris-hydrochloride buffer solution (pH 9.0), and residual activity was measured. Residual activity is normalized with respect to the enzyme activity in the case of no treatment (100%) (refer to Table 5.), indicating that the three kinds of enzymes are extremely stable to surfactants typified by linear alkylbenzenesulfonic acid (LAS). Accordingly, the enzymes are considered to be useful as a detergent component containing surfactants.

Table 5

Surfactant (concentration: 1%)	Residual activity KP43	KP1790	KP9860
free	100	100	100
Na linear alkylbenzenesulfonate (LAS)	100	88	100
Na polyoxyethylene alkylsulfate (ES)	101	102	104
Na dodecyl sulfate (SDS)	104	97	103
Na α-olefinsulfonate (AOS)	100	111	100
Na alkyl sulfate (AS)	113	107	107
α-Sulfofatty acid ester (α-SFE)	112	113	105
Softanol 70H	109	109	104

Treatment conditions: 1% surfactant, 100 mM borate buffer (pH 10.0) 40°C, 4 hours

(8) Effects of oxidizing agents

Each protease was treated at 30°C in 50 mmol/L Britton-Robinson buffer solution containing hydrogen peroxide (pH 8.0), and the residual activity was measured with passage of time. As shown in Fig. 6, KP43 exhibited much greater stability than that of commercially available Savinase or KAP and showed stability as high as that of Durazyme (Novo Nordisk), which was developed by imparting oxidizing agents-resistance to Savinase by use of protein engineering techniques.

(9) Effects of fatty acids

As shown in Table 6, the activity of alkaline proteases of the present invention was not inhibited by oleic acid, one of the components of sebum.

Table 6

Relative activity (%) in the presence of fatty acid

	oleic acid concentration (mM)				
	0	1	2	5	10
KP43 protease	100	100	100	103	119
KP1790 protease	100	100	100	103	121
KP9860 protease	100	100	100	100	106

Example 5 (Cloning of a gene encoding KP9860 protease)

(1) Preparation of genomic DNA of KSM-KP9860

The KSM-KP9860 strain was cultivated in a liquid medium (0.5% glucose, 0.2% Polypepton-S, 0.05% yeast extract, 0.1% $KH_2PO_4 \cdot 7H_2O$, 0.26% $NaCO_3$: pH 9.0) (500 mL) at 30°C for two days,

and the cells were collected by centrifugation. Genomic DNA was prepared from the obtained cells by the method of Saito and Miura (*Biochim. Biophys. Act*, 72, 619(1963)).

(2) Limited proteolysis of KP9860 protease

1) Denaturation of KP9860 protease

KP9860 protease (5 mg/mL)	45 μL
PMSF (100 mM)	20 μL
EDTA (200 mM)	10 μL
SDS (0.08 mg/mL)	25 μL

A protease solution with the above composition was heated in boiling water for 10 minutes. The protease solution was dialyzed against ammonium acetate (2 mM), to thereby remove SDS, EDTA, and PMSF, and was then lyophilized. Subsequently, the lyophilized protease was dissolved in distilled water (100 μL), to thereby serve as a sample of denatured protein.

2) Limited proteolysis by trypsin

Denatured protein sample	100 μL
Trypsin (1 μg/mL, Sigma)	100 μL
1M Tris-HCl (pH 7.5)	50 μL
Distilled water	750 μL

Trypsin was allowed to react against the deratured protein prepared in 1) in an ice bath for 3 hours in the solution with the above composition. After addition of 300 μL of SDS (0.08 mg/mL), 100 μL of EDTA (200 mM) and 200 μL of PMSF (100 mM), limited proteolysis was terminated by heating in boiling water for 3 minutes.

SDS, EDTA, and PMSF were removed through dialysis against ammonium acetate (2 mM), and the solution was lyophilized. Subsequently, the lyophilized was dissolved in distilled water (100 μL), to thereby serve as a sample for SDS-PAGE.

3) Recovering of the partially degraded product

The sample obtained in 2) was subjected to SDS-PAGE with 12% Ready-gel-J (product of Bio-Rad). Protein bands were detected through staining with quick CBB staining solution (product of Bio-Rad). The gel containing the protein band was cut with a razor, and the gel slice was crushed into pieces in a 1.5-mL tube. The buffer for SDS-PAGE (composition: glycine 14.4% (W/V), Tris 3.03%, SDS (product of Bio-Rad) 10%) was added in 5 volumes of the crushed gel, and the mixture was stirred at room temperature, to thereby elute the protein band. The eluate was dialyzed against ammonium acetate (2 mM) and was then lyophilized. The lyophilized sample was served to determine the N-terminal sequence for Protein Sequence type 476A (product of Applied Biosystem).

The obtained N-terminal sequences are shown in Fig. 7.

(3) PCR

20-30 Nucleotides primers for 5'-terminal of + chain and that of the - chain corresponding to the obtained N-terminal sequences were synthesized. PCR reaction was carried out in a 100-μL reaction system by use of a template DNA (100 ng), a primer (20 pmol), and PwoDNA polymerase

(product of Boehringer Mannheim). When inverse PCR was performed, Expand™long template PCR system (product of Boehringer Mannheim) was used in a 50-μL reaction system. PCR carried out by use of these primers, 9860-N2 and 9860-25k-RV, provided a DNA fragment of 527 bp.

(4) Subcloning of the PCR product

The PCR product was purified with a High Pure PCR Product Purification Kit (product of Boehringer Mannheim) and inserted to the *Sma* I site of pUC18 through overnight reaction at 16°C with Ligation kit ver. 2 (product of Takara). The resultant recombinant plasmid and the competent cell *E. coli* JM109 strain (product of Takara) were mixed, and the mixture was subjected to heat shock (42°C, 45 seconds), to thereby transform the *E. coli* JM109 cells. LB was added to the cells. After being maintained at 37°C for one hour, the mixture was applied to an LB plate containing IPTG (0.1 mM, Sigma), X-gal [0.004% (w/v), Sigma], and ampicillin (50 μg/mL, Sigma). Cultivation was performed overnight at 37°C, and grown white colonies were selected as transformants having the recombinant plasmid.

(5) Determination of the nucleotide sequence

The transformant was cultivated overnight at 37°C in LB containing ampicillin (50 μg/mL), and cells were collected through centrifugation. The recombinant plasmid was obtained by use of High Pure Plasmid Isolation Kit (product of Boehringer Mannheim). PCR for sequencing was performed in a 20-μL reaction system by use of a primer and a DNA sequencing

kit (product of PERKIN ELMER), the obtained recombinant plasmid (1 μg) was served as a template DNA. The reaction product was purified by use of Quick Spin Column (product of Boehringer mannheim), and dried up by use of a centrifugal evaporator. The thus-treated sample was subjected to analysis by use of DNA Sequencer Type 377 (product of Applied Biosystem).

The DNA fragment obtained through PCR had the amino acid sequence which matches the N-terminal sequence of the KP-9860 protease, and there were observed sequences, which match common sequences near Asp and His among three amino acids (Asp, His, Ser) forming an active center of alkaline protease such as subtilisin. Thus, the DNA fragment was considered to be a portion of the KP-9860 protease gene.

(6) Southern hybridization

KP9860 chromosome was treated with *Eco*R I, *Sac* I, *Kpn* I, *Hin*d III, *Bam*H I, *Xho* I, *Pst* I, and *Bgl* II. Southern hybridization was performed by use of the obtained 527 bp DNA as a probe, to thereby detect a complementary region.

As a result, hybridization bands were observed in the lanes other than the lane attributed to *Kpn* I.

(7) Inverse PCR

Inverse PCR was performed by use of primers (1 ~ 4 (Fig. 9) Synthesized from the obtained 527 bp sequence. The KP-9860 chromosome was completely digested by use of restriction enzymes, i.e., *Eco*RI, *Hin*dIII, *Pst*I, and *Bgl*II, and each sample was treated by use of Ligation Kit Ver. 2 (product of

Takara)for circularization. Each of the resultant reaction mixtures was served as a template DNA for inverse PCR. PCR reaction (conditions; (94°C-10 seconds, 60°C-30 seconds, 68°C-4 minutes) × 10 cycles; (94°C-10 seconds, 60°C-30 seconds, 68°C-4 minutes + 20 × the number of cycles) × 20 cycles; 68°C-7 minutes; and 4°C-1 minute) was performed by use of the template DNA described above (0.1 μg), primers 1 and 4 (10 pmol, respectively), and the Expand Long Plate PCR System. In addition, PCR (conditions; as described above) was performed by use of the template DNA derived from *Eco* RI digested chromosome (0.1 μg), primers 2 and 3 (10 pmol, respectively), and the Expand Long Plate PCR System. The resultant amplified DNA fragments were purified by use of High Pure PCR Product Purification Kit, and terminals were converted to blunt-ended by use of DNA Blunting Kit (product of Takara). Each of the obtained DNA fragments and *Sma*I digested pUC18 were mixed, and the mixture was treated with Ligation Kit Ver. 2. As described above, *E. coli* JM 109 strain was transformed by the recombinant plasmid, and the obtained recombinant plasmid was served as a template DNA for sequencing. Thus, the nucleotide sequence of the amplified DNA fragments was determined.

(8) Analysis of the entire nucleotide sequence of the KP-9860 protease gene

The sequencing revealed that the KP-9860 protease gene contains an open reading frame (ORF) encoding the 1917 bp, 639 amino acid residues and that the ORF contains a region

(NDVARHIVKADVAQSSYGLY) which matches the N-terminal sequence of the purified KP9860 protease. Judging from the N-terminal sequence, the muture region of KP9860 protease gene was deduced to be the 1302 bp, encoding 434 amino acid residues (Sequence No. 3, molecular weight 45310 Da). Upstream of the ORF, there were observed sequences which are deduced to be a promoter region (-35 region: ttgtgt, -10 region: tacgat) and a ribosome-binding site (SD sequence: aggagt). Downstream of the termination codon (taa), there was an inverted repeat having a free energy of -26.2 kcal/mol, which is deduced to be a terminator.

The procedure of Example 5 was repeated, to thereby analyze the entire nucleotide sequence and amino acid sequence of each of the genes of KP-43 protease and KP-1790 protease. The results are shown in Sequence Nos. 4 and 5.

Example 6

Washing Test:

A washing test was carried out according to JIS K 3371. Detergents whose compositions are shown in Table 7 were dissolved in water containing 71.2 mg of $CaCO_3$/L (4° DH) so as to adjust the concentration, and each protease was added to detergent solution so as to adjust the concentration of the alkaline protease to 40 mAPU/L according to the Anson-Hemoglobin method (see Table 8).

Collars of shirts (worn for 3 days) were employed as specimens. For comparison, after the cloth of a collar was

cut into a size of about 8 × 8 cm, the cloth was washed at 15°C and 100 rpm, for 10 minutes by use of a Terg-O-Tometer (Ueshima Seisakusyo) with addition of the enzyme or without addition of the enzyme. After being rinsed and dried, pairs of collar clothes (15 pairs) were compared and evaluated by visual judgement. When the soil was almost completely cleaned, an evaluation of 5 was assigned, and when the soil was hardly cleaned, an evaluation of 1 was assigned, and the total scores of 15 specimens were calculated. The detergency index was expressed as the scores of each composition, with the detergency of a detergent composition without addition of the enzyme taken as 100. The results are shown in Table 8.

Table 7

(wt.%)

Compound (%)	Detergent A	Detergent B	Detergent C
LAS	23.0	4.0	20.0
AS	4.0		
AE	5.0		
AEP		5.0	
AES		20.0	
Fatty acid salt	3.0	2.5	2.0
Zeolite	22.0		20.0
Sodium carbonate	15.0		
Potassium carbonate	3.0		
Amorphous silicate	7.0		7.0
Crystalline silicate	4.0		
Sodium sulfite	2.0	0.5	2.0
Sodium sulfate	2.0		23.0
AA-MA	5.0		
Citrate			10.0
PEG	2.0		2.0
Monoethanolamine		8.0	
Ethanol		5.0	
Water	3.0	balance	7.0
Form	G*	L**	G*
Concentration in use	20g/30L	20g/30L	40g/30L
pH after washing	10.7	9.2	8.0

*) G stands for granular.
**) L stands for liquid.
LAS: sodium linear alkyl(C12-C14)benzene sulfonate (free acid incorporated into a liquid detergent)
AS: alkyl sulfate
AE: polyoxyethylene lauryl ether (average EO addition of 4 moles)
AEP: polyoxyethylene polyoxypropylene lauryl ether (average EO addition of 8 mol, average PO addition of 3 mol)
AES: alkyl ether sulfate (average EO addition of 2.5 mol)
Fatty acid: palm oil-derived fatty acid sodium salt
Zeolite: zeolite 4A, average particle size of 3 μm
Sodium carbonate: dense ash
Amorphous silicate: JIS No. 2 sodium silicate
Crystalline silicate: pulverized SKS-6 (product of Hoechst Tokuyama), average particle size of 15 μm
AA-MA: Sokalan CP5, acrylic acid-maleic acid copolymer (product of BASF)
PEG: polyethylene glycol, average molecular weight of 8,000

Table 8

	Protease	Detergency index
		Detergent A
Detergent of the invention 1	*Bacillus sp.* KSM-KP43 (Example 2)	106
Detergent of the invention 2	*Bacillus sp.* KSM-KP1790 (Example 3)	106
Detergent of the invention 3	*Bacillus sp.* KSM-KP9860 (Example 3)	105
Comparative detergent 1	Savinase 120T type White® (Novo Nordisk)	103.5
Comparative detergent 2	Durazym 6.0T® (Novo Nordisk)	103.5
Comparative detergent 3	None	100

Table 8 demonstrates that, even under the same activity conditions, the detergent composition containing the enzyme of the present invention (detergent A) exhibits superior detergency as compared to detergents containing conventional proteases. Detergents B and C also exhibit excellent detergency of the present invention.

Example 7

A granular product was prepared through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 by use of a purified sample of protease of the present invention which had been derived from *Bacillus sp.* KSM-KP43, KSM-KP1790, or KSM-KP9860 and prepared in Example 2 or 3. The granular product (6 APU/g) (1 part by weight) was incorporated into each of detergents (100 parts by weight) having compositions shown in Table 9, to thereby obtain

detergent compositions of the present invention. When the detergent was of the granular type, such a detergent was prepared by blending a granular detergent base which is free of components; i.e., an enzyme, PC, AC-1, and AC-2, with a granulated enzyme, granulated PC, granulated AC-1, and granulated AC-2. Each detergent was dissolved in water containing 71.2 mg $CaCO_3$/L (4° DH) at a concentration for use, and a collar was washed in a manner as described in Example 6. The detergents produced herein exhibit excellent washing power, and are useful for a laundry detergent.

Table 9

Component (%)	Detergents of the present invention									
	4	5	6	7	8	9	10	11	12	13
LAS-2	20		20.5		12				5	10
LAS-3		15								
AS-2			5		10		20			
SAS	3									
AOS		3								
SFE		8								
Fatty acid salt	2	6	4	10	3	3	2	1.5		
AES-2								20		
AE-3	3									10
AE-4		3	3	15		15	3		15	
AE-5							2	20	20	25
AG									5	7
Zeolite	30	18	15	15		10	20			
Oil-absorbing carrier				10		12				
Crystalline silicate				20						
Amorphous silicate	12	1	8		10		5			
STPP					25.5	20				
Sodium carbonate	10	27	25	10	10	15	17.5	0.1		
Potassium carbonate		3		2	5					
Sodium sulfite	2	2			1			0.2	0.2	0.2
Sodium sulfate	4.5	1.5		1	11	8	10			
Sodium citrate			4	2			5	1.5	1	1
NTA						2				
Monoethanol -amine								4	5	6
PAA					1	1.5	3			
AA-MA		3	3	5						
CMC	2									
PEG	5	2	2	2	2			1.5		
PVP							2			
Fluorescent dye	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1
Perfume	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
Water	4	5	3	0.5	6	1	5	43.7	38.2	30.2
Ethanol								5	5	5
Propylene glycol								2	5	5
Enzyme	2	2	2	3	3	2	2	0.1	0.2	0.2

Component (%)	Detergents of the present invention									
	4	5	6	7	8	9	10	11	12	13
PC			3	3	10	3				
AC-1			2							
AC-2				1						
Total	100	100	100	100	100	100	100	100	100	100
Form	G*	G*	G*	G*	G*	G*	G*	L**	L**	L**
Concentration in use	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20mL/30L	20mL/30L	20mL/30L

*) G stands for granular.
**) L stands for liquid.
LSA-2: alkylbenzene sulfonic acid (C10-C14 alkyl chain) which was neutralized with 48% NaOH
LSA-3: alkylbenzene sulfonic acid (C10-C14 alkyl chain) which was neutralized with 50% NaOH
AS-2: sodium salt of Dovanol 25 sulfate (C12-C15 sulfate)
SAS: sodium C13-C18 alkane sulfonate
AOS: sodium α-olefin sulfonate
SFE: sodium salt of palm oil α-sulfofatty acid methyl ester
Fatty acid salt: sodium palmitate
AES-2: sodium polyoxyethylene alkyl (C12-C15) ether sulfate (average EO addition of 2 moles)
AE-3: EO adduct (average 3 moles) of C12-C13 alcohol
AE-4: EO adduct (average 7.2 moles) of C12-C15 alcohol
AE-5: EO adduct (average 7 moles) of C12-C15 secondary alcohol
AG: alkyl (palm oil-derived) glucoside (average polymerization degree of 1.5)
Oil-absorbing carrier: Amorphous sodium aluminosilicate, oil absorption of 235 mL/100 g
Crystalline silicate: SKS-6 (δ-$Na_2Si_2O_5$, crystalline layered silicate, average particle size of 20 μm)
Amorphous silicate: JIS No. 1 sodium silicate
STPP: sodium tripolyphosphate
NTA: sodium nitrilotriacetate
PAA: sodium salt of poly(acrylic acid), average molecular weight of 12,000
AA-MA: acrylic acid/maleic acid copolymer
CMC: carboxymethyl cellulose sodium
PEG: polyethylene glycol, average molecular weight of 6,000
PVA: polyvinylpyrrolidone, average molecular weight of 40,000,

K value of 26-35
Fluorescent dye: Tinopal CBS and Whitex SA (1 : 1 (wt.)), only Cinopearl incorporated into a liquid detergent
Perfume: A perfume composition disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 8-239700
Enzyme: Lipolase 100T, Termamyl 60T, and KAC 500® (product of Kao Corporation) 1 : 1 : 1 (wt.)
PC: sodium percarbonate, average particle size of 400 μm, coated with sodium metaborate
AC-1: tetraacetylethylenediamine
AC-2: sodium lauroyloxybenzene sulfonate

Example 8

Among the components shown in Table 10, sodium percarbonate and sodium carbonate (dense ash) were mixed with stirring. To the mixture, a 40% aqueous solution of sodium polyacrylate and sodium linear alkylbenzene sulfonate (or nonionic surfactant or sodium lauroyloxybenzene sulfonate) were added. Subsequently, a granulation product of alkaline protease which had been derived from *Bacillus sp.* KSM-KP43 and prepared in Example 7 was added to the mixture. The resultant mixture was homogeneously stirred, to thereby prepare a bleaching agent. A collar was immersed in a 0.5% aqueous solution of each of the bleaching agents at 20°C for 30 minutes, and subsequently washed with detergent A (Example 6) in a Terg-O-Tometer at 100 rpm for 10 minutes at 20°C. The obtained bleaching agents have excellent bleaching ability, and are useful as a bleaching agent for laundry.

Table 10

(wt.%)

Component	Bleaching agents of the present invention			
	14	15	16	17
Sodium percarbonate[1)]	80.0	80.0	80.0	80.0
Sodium carbonate (dense ash)	16.0	12.0	16.0	12.0
Anionic surfactant[2)]	2.0	2.0	-	-
Nonionic surfactant[3)]	-	-	2.0	2.0
Sodium polyacrylate[4)]	1.0	1.0	1.0	1.0
Sodium lauroyloxy-benzene sulfonate	-	4.0	-	4.0
Bacillus sp. KSM-KP43 Alkaline protease (Ex.7)	1.0	1.0	1.0	1.0

1) Particle size: 500-700 μm
2) Sodium linear alkylbenzene sulfonate (C12-C14)
3) Polyoxyethylene alkyl ether (C12-C14 alkyl, average EO addition of 12 mol)
4) Average molecular weight of 8,000

Example 9

The procedure of Example 8 was repeated, to thereby prepare detergent compositions for an automated dishwasher having a composition shown in Table 11. Washing power of the obtained compositions was tested under the following conditions. The obtained detergents have excellent washing power, and are useful as a detergent for an automated dishwasher.

Table 11

(wt.%)

Component	Detergents of the present invention			
	18	19	20	21
Pluronic L-61[1]	4	-	4	4
Softanol EP-7085[2]	-	4	-	-
Trisodium citrate	30	30	-	-
EDTA	-	-	30	-
Sodium tripoly-phosfate	-	-	-	30
Sodium percarbonate	20	20	20	20
Sodium carbonate (dense ash)	20	20	20	20
Amorphous silicate[3]	10	10	10	10
AA-MA[4]	4	4	4	4
Sodium sulfate	10	10	10	10
Lipolase 100T® (Novo Nordisk)	0.5	0.5	0.5	0.5
Termamyl 60T® (Novo Nordisk)	1	1	1	1
Bacillus sp. KSM-KP43 alkaline protease (Ex. 7)	0.5	0.5	0.5	0.5

1) Polyoxyethylene-polyoxypropylene copolymer (average molecular weight of 2,000)
2) Ethylene oxide (7 moles) and propylene oxide (8.5 moles) adduct of C12-C14 sec-alcohol
3) JIS No. 2 sodium silicate
4) Acrylic acid-maleic acid copolymer

(1) Preparation of a soiled dish

Egg yolk (2.5 g) was homogeneously brushed onto one ceramic dish having a diameter of 25 cm. The dish was dried in a drier at 115°C for 60 minutes.

(2) Washing conditions

Washer used; Full automated dishwasher (NP-810, product of Matsushita Electric Industry Co., Ltd.)

Type of washing; Standard course

Water for washing; Hardness of 62.3 mg $CaCO_3$/L (3.5° DH)

Concentration of detergent; 0.2 wt.%

(3) Method for evaluation

Five soiled dishes were washed in the washer under the above conditions by use of the detergent compositions of Example 9. The washed dish was stained with a 1% Erythrosine solution, to thereby color residual protein. The degree of protein soil was judged visually.

Example 10

Detergent compositions for an automated dishwasher were obtained from components shown in Table 12. Washing power of these compositions were evaluated through a test similar to that of Example 9. The compositions provided an excellent washing effect.

Table 12

(wt.%)

Component		Detergent compositions of the present invention				
		22	23	24	25	26
(a)	Sodium carbonate	30		30		50
	Sodium hydrogen-carbonate		25		25	
(b)	Sokalan CP5[1)]	5	6	5	5	5
(c)	Sodium hydrogen-percarbonate	5		6		
(d)	Limonene	2	2		1	1
	Softanol EP7045[2)]			2	1	1
(c)	Amorphous sodium aluminosilicate (Synth. Ex.1)[3)]	2		2	1	3
	Amorphous sodium aluminosilicate (Synth. Ex.2)[4)]		2		1	
Lipolase 100T® (Novo Nordisk)		0.5	0.5	0.5	0.5	0.5
Termamyl 60T® (Novo Nordisk)		1	1	1	1	1
Bacillus sp. KSM-KP43 alkaline protease (Ex.7)		0.5	0.5	0.5	0.5	0.5
Sodium malate			10		5	
Sodium citrate		15		10	4	8
Sodium sulfate		39	53	43	55	30

1) Acrylic acid/maleic acid copolymer (product of BASF)
2) Ethylene oxide (7 moles) and propylene oxide (4.5 moles)adduct of C12-C14 sec-alcohol
3), 4) Synthetic Example disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 6-179899

Example 11

Enzymes were added to the above-described detergent A (Example 6) in amounts shown in the following Table 13. A collar portion of a white shirt was washed in a manner similar to that of Example 6.

Table 13

(wt.%)

Enzyme	Detergents of the present invention						
	27	28	29	30	31	32	33
Protease of the present invention[1)]	-	0.5	0.5	0.5	0.5	0.5	0.5
Conventional protease[2)]	-	-	0.6	-	-	0.6	0.6
Cellulase[3)]	-	-	-	0.7	-	0.7	0.7
Lipase[4)]	-	-	-	-	0.5	-	0.5

1) A granular product prepared through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 by use of a purified sample of protease of the present invention which was derived from *Bacillus sp.* KSM-KP 43 strain and prepared in Example 2 (6 APU/g)
2) Protease K-16 disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 5-25492 which was modified to have 5 APU/g through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990
3) KAC-500® (cellulase, 500 U/g, product of Kao Corporation)
4) Lipolase 100T® (product of Novo Nordisk)

The results clearly show that the combination of the protease of the present invention and a conventional protease, cellulase, or lipase enhances a washing effect.

Industrial Applicability

The alkaline protease of the present invention has excellent stability against a variety of surfactants; resistance to fatty acids; and high stability against an oxidizing agent, and is therefore useful as an enzyme for a detergent for an automated dishwasher and for a laundry detergent, both containing a bleaching component.